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Amino acid sequence of an iodotyrosyl-containing peptide produced by tryptic digestion of thyroglobulin

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AMINO ACID SEQUENCE OF AN IODOTYROSYL-CONTAINING PEPTIDE PRODUCED BY TRYPTIC DIGESTION OF THYROGLOBULIN

By

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THESIS

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ABSTRACT

Thyroglobulin was digested with trypsin. An iodotyrosyl-containing peptide was isolated from tryptic digests by a combination of electrophoresis and paper chromatography. The amino acid sequence of this peptide was determined by applying the Edman degradation and by using the proteolytic enzyme, carboxypeptidase A. The sequence of this peptide is:

NH_2-Ala-Ile(Leu)-Ala-Ser-Phe-Gly-Asp-Tyr-Glu-Phe-COOH .

The importance of the position of the tyrosyl residue which is iodinated to form monoiodotyrosine (MIT), diiodotyrosine (DIT), and thyroxine (T_4) is considered in connection with the structure-activity relationship of the protein.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Although nothing is known at the present time about the sequence of the amino acids in thyroglobulin, the amino acid composition of thyroglobulin has been determined. Tyrosine values for various species, after hydrolysis, range between 3.0 and 3.5 per cent, which corresponds to 110 to 128 residues per mole of protein. The iodoamino acids formed by the iodination of thyroglobulin are diiodotyrosine, monoiodotyrosine, and thyroxine. Iodination studies have shown that most of the iodine is incorporated as diiodotyrosine; however, smaller yields of monoiodotyrosine and thyroxine were obtained. The native three dimensional configuration of thyroglobulin does not appear to be essential for the synthesis of thyroxine *in vitro*, the principal parameter still to be evaluated is probably the length of the polypeptide chain required for its synthesis.

Since during *in vivo* iodination only a very small percentage of the tyrosine in thyroglobulin is iodinated, it is possible that the amino acid sequence plays an important role in this process. A knowledge of the sequence of an iodotyrosyl-containing peptide is required in order to carry out peptide synthesis and enzymatic iodination studies. It is the purpose of this thesis to present the isolation, amino acid composition, and the sequence of

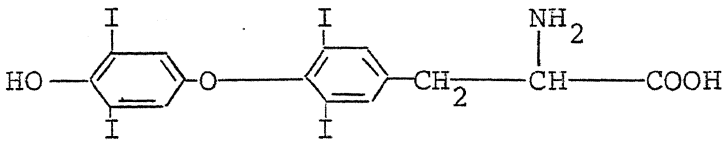
amino acids of an iodotyrosyl-containing peptide produced by tryptic digestion of thyroglobulin.

II. LITERATURE REVIEW

A. Thyroglobulin

Thyroglobulin is the main component of the colloid that is secreted into the lumen of the follicles by the surrounding cells of the thyroid. This specific iodoprotein has a molecular weight of about two-thirds of million (Edelhoch, 1960; Lundgren and Williams, 1939). It contains 4 per cent carbohydrate, 3 per cent tyrosine, and iodine in amounts varying from 0.1 to 0.9 per cent of the total molecular weight (Edelhoch and Rall, 1964). The amino acid composition of calf thyroglobulin (Peiz and Morris, 1960) is given in Table I.

In addition to the amino acids commonly found in proteins, thyroglobulin contains a number of iodinated amino acids that exhibit hormonal activity when released by proteolysis. The most potent of these is thyroxine (T_4).



Thyroxine (T_4)

The iodoamino acid content of thyroglobulin is known to vary considerably. The iodoamino acid content of beef

TABLE I

THE AMINO ACID COMPOSITION OF CALF THYROGLOBULIN

Amino Acid	Per cent by Weight	Moles/M.Wt.(670,000)[a]
Aspartic acid	6.36	371
Threonine	3.68	244
Serine	6.28	484
Glutamic acid	12.57	653
Proline	5.45	376
Glycine	3.40	400
Alanine	4.67	440
Half-cystine[b]	3.08	202
Valine	4.52	306
Methionine	0.90	46
Isoleucine	2.21	131
Leucine	7.98	473
Tyrosine	3.07	126
Phenylalanine	5.69	259
Lysine	2.49	130
Histidine	1.22	60
Arginine	7.51	322
Amide N	----	538
Glucosamine	2.30	96

a. Assumed molecular weight

b. This value includes only cystine in disulfide linkage

thyroglobulin (Robbins, 1963) is given in Table II.

Table II shows that only a small percentage of the tyrosines which occur in thyroglobulin are iodinated. If it is assumed that both of the tyrosines that form T_4 are part of the protein molecule, then 17.5% are iodinated. If the opposite assumption is made, then only 15% are iodinated. If random iodination were occurring, one would expect a higher percent iodination.

It is now well known that 19 S thyroglobulin is not a homogeneous protein but consists of a variety of molecules with different iodine contents (Ui, et. al., 1961; Ingbar, et. al., 1959; Edelhoch and Metzger, 1961; Edelhoch and Lippoldt, 1962). Edelhoch and his co-workers have observed the formation of a slower-sedimenting component when they treated a solution of calf thyroglobulin with alkali (Edelhoch, 1960), heat (Edelhoch and Metzger, 1961) or with various reagents including anionic detergents (Edelhoch and Lippoldt, 1960), urea and guanidine hydrochloride (Edelhoch and Lippoldt, 1964). From the measurement of sedimentation velocity, viscosity, diffusion and light scattering, it was inferred that the slower-sedimenting component had one-half the molecular weight of the native molecule. In combination with the results on the reductive cleavage of disulfide bonds in this protein (DeCrombrugghe, et. al., 1966; Edelhoch and DeCrombrugghe, 1966; DeCrombrugghe and Edelhoch, 1966), they proposed a

TABLE II

IODOAMINO ACID CONTENT OF BEEF THYROGLOBULIN

Component	Moles/670,000
Monoiodotyrosine (MIT)	10
Diiodotyrosine (DIT)	6
Thyroxine (T_4)	3
Total iodine (% of protein)	0.7

molecular model of thyroglobulin in which a 19 S molecule consists of two equal subunits, each made up of two polypeptide chains linked together by a few disulfide bonds.

Studies on the biosynthesis of thyroglobulin (Lissitzky, et. al., 1964; Seed and Goldberg, 1965; Nunez, et. al., 1965; Sellin and Goldberg, 1965) have demonstrated that precursor proteins with $S_{20,w}$ of 3 to 8 S and 12 S are involved and that iodination of thyroglobulin occurs after the synthesis of polypeptide chains is completed.

Recent studies on the subunit structure of hog thyroglobulin by Tarutani and Ui (1969) have shown that at lower concentrations of sodium dodecyl sulfate, thyroglobulin showed the presence of a slower-sedimenting component (12 S) together with the unaltered protein (19 S) on analytical ultracentrifugation. By increasing sodium dodecyl sulfate concentrations, the conversion of 19 S and 17 S occurred, giving a mixture of 12 S to 17 S. Molecular weight determinations by means of sedimentation equilibrium have revealed that the 12 S component has a molecular size approximately half that of native 19 S thyroglobulin (M. Wt. 670,000). 17 S protein was estimated to have the same size as that of 19 S. Furthermore, under all conditions studied, neither a complete dissociation into 12 S nor further dissociation of 12 S into smaller units was observed. Therefore, they concluded that there are two types of molecules in native hog thyroglobulin (19 S).

One consists of two equal subunits (12 S) which are linked by noncovalent bonds, and the other in which all the polypeptide chains are linked by covalent bonds and/or by other similarly strong forces.

Denaturation of thyroglobulin, defined as insolubility at the isoelectric pH, is produced by acid (pH<4.5) (Heidelberger and Palmer, 1933; Litonjua, 1961; Heidelberger and Pedersen, 1935) or alkaline pH (>11.3) (Edelhoch and Metzger, 1961; Heidelberger and Pedersen, 1935; Metzger and Edelhoch, 1961) as well as by heating (>53°) (Metzger and Edelhoch, 1961). The rate of denaturation is increased by either detergent or concentrated urea (or guanidine) solution (Edelhoch and Lippoldt, 1960). Extensive in vitro iodination also results in denaturation (Edelhoch and Lippoldt, 1962).

It has been shown that the ionized form of tyrosine is iodinated approximately a million times more rapidly than the unionized form (Li, 1942). Consequently, tyrosyl residues which ionize normally should be more readily iodinated than those that ionize abnormally. Roche, et. al., (1947) and Edelhoch (1962) found that when thyroglobulin is iodinated, most of the iodine is incorporated as diiodotyrosine; however, smaller yields of monoiodotyrosine and thyroxine were obtained. Edelhoch (1962) also found that the formation of thyroxine was largely unaffected when iodination occurred in 8 M urea. Thus, it

seems that a unique three dimensional configuration of the native structure of thyroglobulin is not necessary for the *in vitro* synthesis of thyroxine. Moreover, the *in vitro* synthesis of thyroxine is not unique to thyroglobulin since it is also formed by iodination of casein and insulin (Roche, et. al., 1949). Although protein conformation does not appear to be important, the length of the polypeptide chains is of consequence. Tryptic, peptic and chymotryptic digests of thyroglobulin when iodinated, under identical conditions failed to show synthesis of thyroxine although normal amounts of monoiodotyrosine and diiodotyrosine were formed.

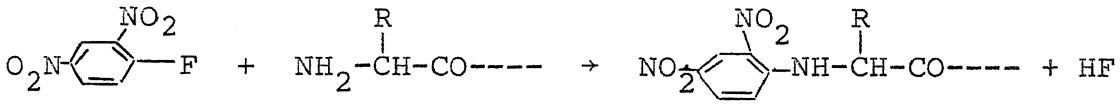
B. Sequence Determination of Peptide Chains

The approach to sequence determination of peptide is to determine the terminal amino acids of the peptide and either sequentially cleave amino acids or work with small peptides and fit the peptides back together by overlap. Terminal residues are of two types: $\overset{+}{N}H_3\text{-}\overset{R}{\underset{|}{C}}H\text{-}CO\text{----}$, N-terminal residues, and $\text{----}NH\text{-}\overset{R}{\underset{|}{C}}H\text{-}CO_2^-$, C-terminal residues.

1. Methods of amino-terminal amino acid determination.

a. Dinitrophenyl (DNP) method. This method was introduced by Sanger (1945). It depends on the fact that (a) 1-fluro-2, 4-dinitrobenzene reacts quantitatively under

mild alkaline conditions with terminal α-amino groups of peptides (and the ε-amino groups of lysine residues) to form "DNP-peptides"



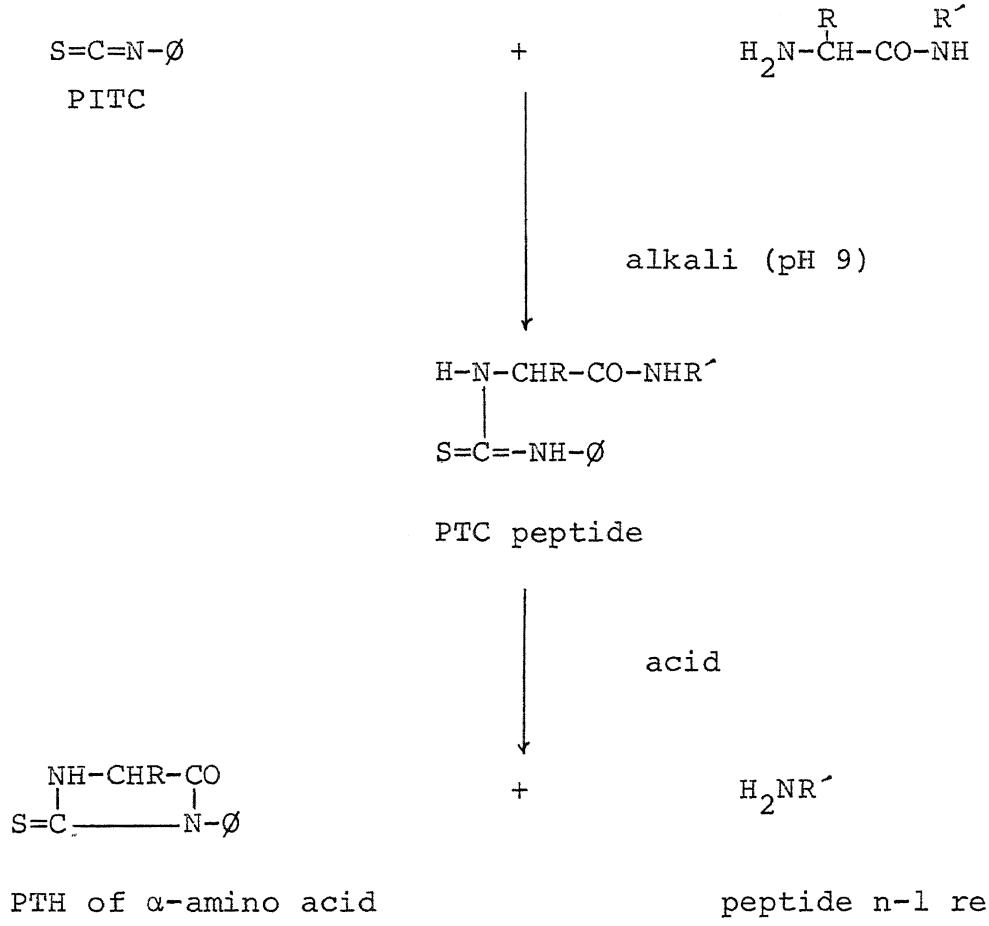
1-fluoro-2,4 dinitrobenzene

DNP-peptides

and (b) the Ø-N bond formed is stable to acid hydrolytic attack. The DNP-peptides are, therefore, completely hydrolysed yielding a mixture of amino acids and DNP-amino acids corresponding to the N-terminal residues. These DNP-amino acids are bright yellow in color and can be extracted into organic solvents. Chromatographic determination of the amino acid involved reveals the nature of the N-terminus. This method involves degradation of the peptide system and can therefore only be used once for a given peptide sample.

b. Phenylthiocarbamyl method. This method was devised by Edman (1950). The reagent phenylisothiocyanate (PITC) reacts with the free α-amino group in dilute alkali to form the phenylthiocarbamyl (PTC) derivatives. The PTC compound, in the presence of acid, cyclizes to form a

phenylthiohydantoin (PTH derivatives). If a PTC peptide is formed from the starting compound, then the terminal peptide bond is so labilized that the end amino acid is split off during the acid incubation which is necessary for the cyclization to occur.



This method does not involve any degradation of the peptide system other than the splitting out of the N-terminal amino acid residue. It can, therefore, be repeated on a given specimen, in principle at least, to split out each

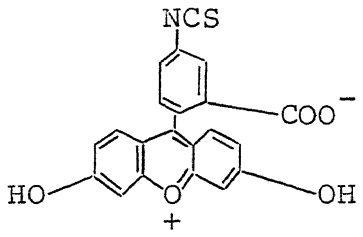
successive N-terminal amino acid residue and thus determine the complete "left-to-right" sequence of residues.

c. Fluorescein isothiocyanate method. This new micro-analysis of the N-terminal amino acid of proteins and peptides using fluorescein isothiocyanate isomer I was described by Maeda and Kawauchi (1968). The reactions are closely related to those involved in the use of phenylisothiocyanate (Edman, 1950). The reaction sequence is shown on page 13. This method is capable of detecting very low levels of peptide (at least 10^{-2} to 10^{-3} of that for dinitrophenylation), and at the same time, it can be used for a sequence analysis of a peptide chain, as is the case with Edman's phenylisothiocyanate method.

The FTH-amino acids possess a yellowish color, and upon UV irradiation fluoresce just as an FTC-protein. Thus, their locations on the chromatogram are easily detected.

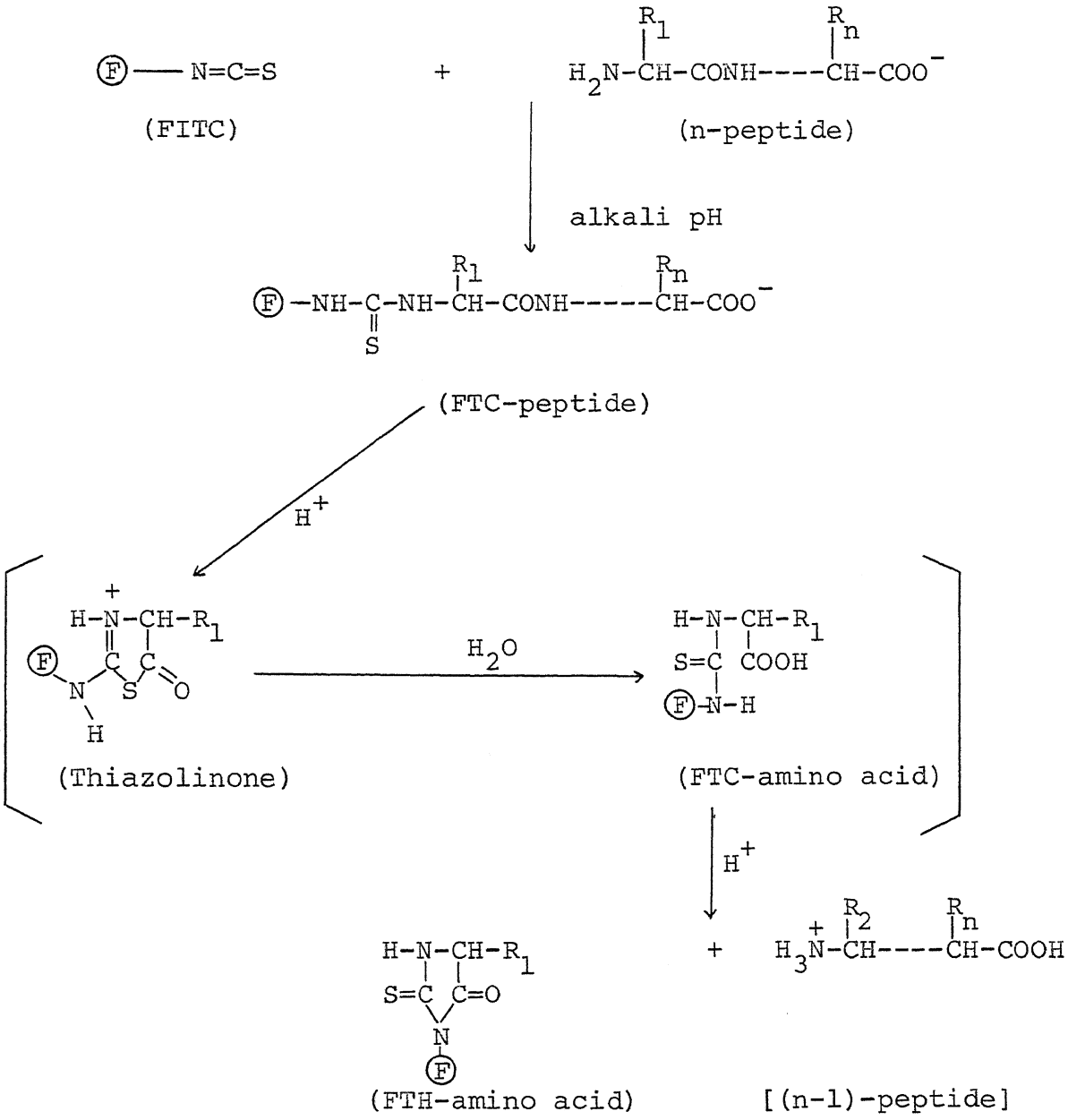
d. Leucine aminopeptidase method. This enzyme is applicable to N-terminal amino acid determinations. It requires the presence of a free α-amino function for activity, and, hence, splits only the N-terminal amino acid from the polypeptide chain.

e. Dansyl method. (Gary and Hartley, 1963). Determination of the N-terminal residues of proteins and peptides can be carried out using 1-dimethylaminonaphthalene-5-sulphonyl chloride (DNS-Cl), which reacts with free



Ⓕ indicates Fluorescein Chromophore

Structure of Fluorescein-isothiocyanate (FITC) Isomer I

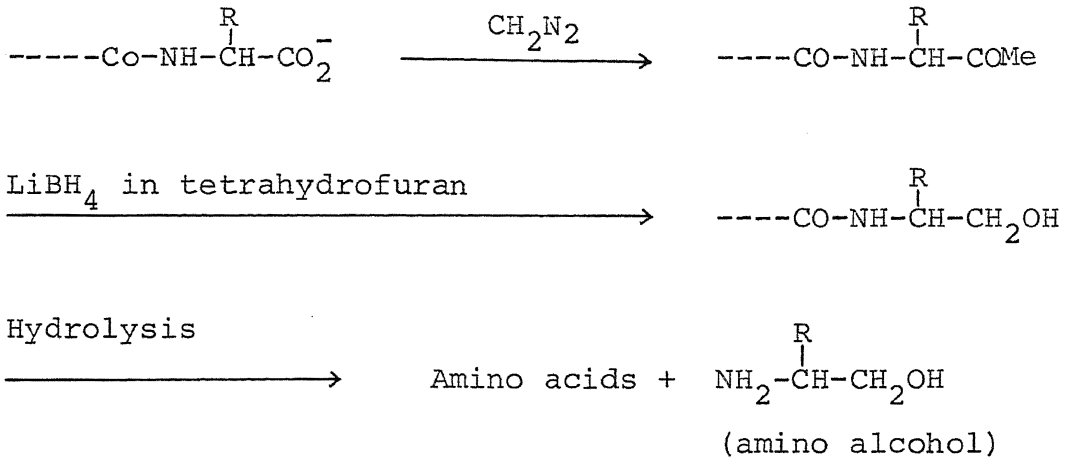


amino and phenolic groups. The derivatives formed (DNS-X) are very resistant to acid hydrolysis, and have an intense yellow fluoresence, making the method applicable to 10^{-4} to 10^{-3} μmole of peptide. It is thus about 100 times more sensitive than the fluorodinitrobenzene method.

2. Methods of carboxyl-terminal amino acid determination.

a. Carboxypeptidase method. Carboxypeptidase, which splits off preferentially the C-terminal amino acid from a peptide chain, has been used by Lens (1949) for C-terminal identification. As the C-terminal residue is being hydrolyzed, the enzyme does not stand by until this residue is completely removed before starting its hydrolytic action on the next, newly exposed C-terminal residue, but attacks the latter, and likewise the still newer C-terminal residue, until a C-terminal residue is finally reached which is resistant to the enzyme and further action ceases.

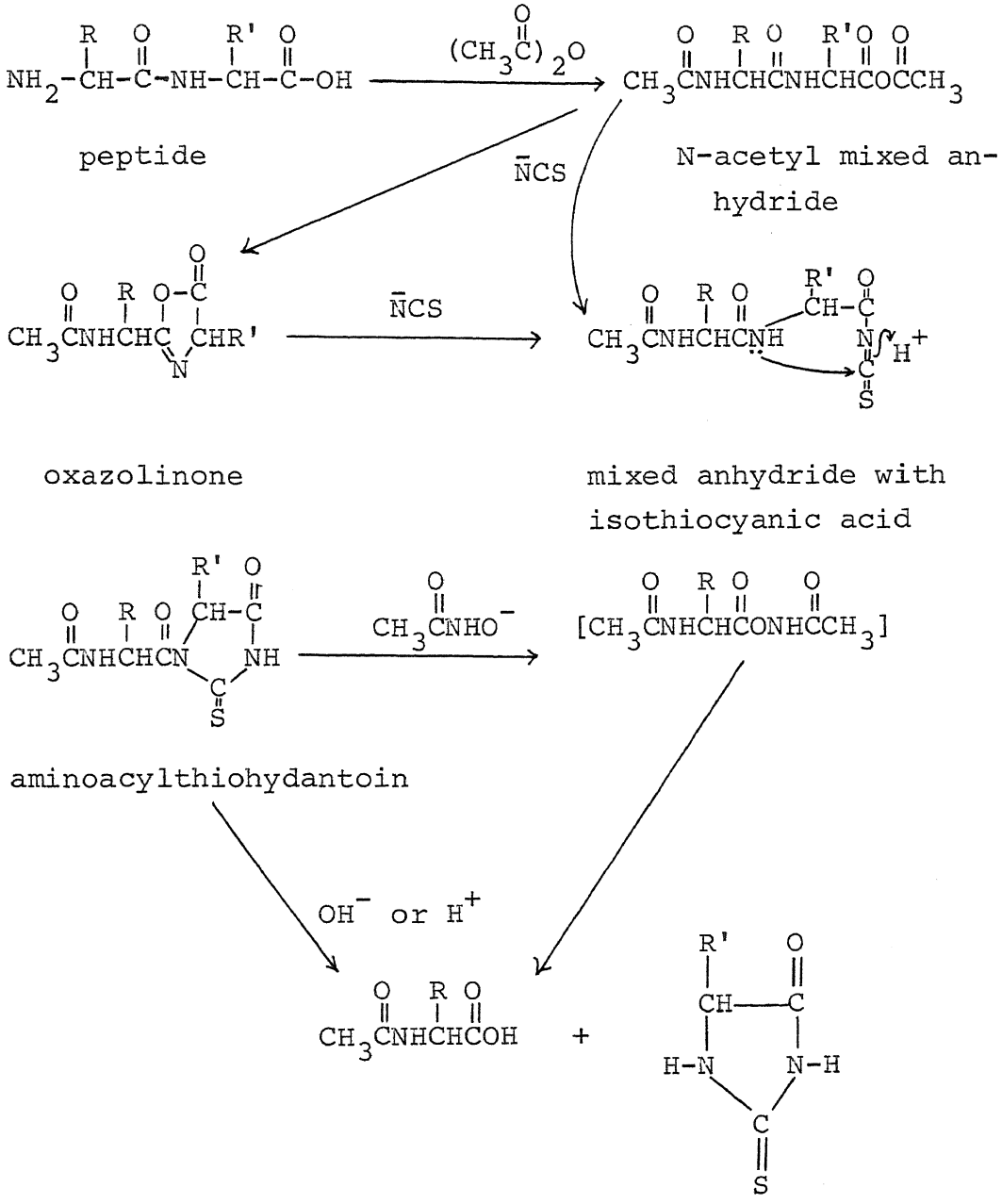
b. Amino alcohol methods. Three very similar methods have been described in which the C-terminal amino acid residue is converted into the corresponding amino alcohol. (a) Chibnall and Rees (1951) have used the following series of reactions:



The amino alcohol can be separated chromatographically or by eletrodialysis and estimated by periodate oxidation. (b) Fromageot, et. al., (1950) reduced the free carboxyl group directly with lithium aluminum hydride in 4-ethyl-morpholine. (c) Wessely, et. al. (1952) converted the carboxyl group to acid chloride with thionyl chloride and reduced with sodium borohydride.

c. Ammonium thiocyanate method (Stark, 1968). This sequential degradation of amino acids from the C-terminus of a peptide has been carried out by use of ammonium thiocyanate and acetic anhydride. The peptide thiohydantoins formed are cleaved with acetohydroxamic acid under mild condition.

This method is limited in that carboxyl-terminal aspartic acid and proline are not removed, but all other residues, including asparagine, glutamine, and glutamic acid, do form acylthiohydantoins and are degraded.


peptide
N-acetyl mixed anhydride
oxazolinone
mixed anhydride with isothiocyanic acid
aminoacylthiohydantoin
OH⁻ or H⁺
+

III. EXPERIMENTAL

A. Proteins

The proteins used in this work are as indicated: Thyroglobulin: Porcine, type II; Trypsin: twice crystallized, dialyzed and lyophilized, substantially salt free, Bovine pancreas type III; Carboxypeptidase A: dialyzed and recrystallized, DFP-treated, aqueous suspension (58 mg/ml). All of these proteins were purchased from the Sigma Chemical Company, St. Louis, Missouri.

B. Tryptic Digestion of Thyroglobulin

Thyroglobulin (14 mg) was placed in a test tube and 2.8 ml of 0.1 M Tris buffer, pH 8.0, added. Digestion was performed at 30° for 24 hours with 2.8 mg of crystalline trypsin. The reaction was stopped by the addition of 0.93 ml of 40% trichloroacetic acid (TCA). The precipitate was removed by centrifugation and the supernatant was washed three times with equal volumes of peroxide-free ether. The ether phase was discarded and the trichloroacetic acid supernatant (ca. 3 ml) was used as the sample for isolation of the iodopeptide.

C. Isolation of the Iodopeptide

The TCA supernatant (200λ) was applied in bands to two sheets of Whatman No. 3 MM filter paper (2 x 8 inches). The sheet was subjected to electrophoresis in pyridine-

acetate buffer, pH 6.8 (40 ml of pyridine was diluted to 1000 ml with distilled water and the pH adjusted to 6.8 with acetic acid). The potential gradient was 10 to 15 V/cm for 4 hours. The paper strips were dried in a current of air and examined for iodopeptide under a short wave ultra-violet light (Model SL 2537, Ultra-Violet Products, Inc., South Pasadena, California). Those fractions having more than one component were further purified by paper chormatography. For this purpose, the peptide was cut out and sewed on a Whatman No. 3 MM filter paper strip (1 x 15 inch). Separation was accomplished by ascending chromatography in a solvent system of n-butanol-acetic acid-water (4:1:1 v/v) for 18 hr. The peptide band was eluted with distilled water. The eluates were evaporated to dryness in a rotary evaporator.

D. Identification of Iodotyrosyl-Containing Peptide

The iodopeptide residues were dissolved in an aliquot of distilled water and applied to Whatman No. 1 filter paper (10 x 10 inch) along a line one inch from the bottom edge of the paper and at intervals of one inch. Chromatography was carried out in a solvent system of ethanol-ammonium hydroxide (0.88 M)-water (90:5:5 v/v) for 6 hr. The chromatograms were treated with ceric-arsenite reagent (Bowden, et. al., 1955): (1) ceric sulfate, 10% in 1 N H_2SO_4, (2) sodium arsenite, 5% in 1 N H_2SO_4. A mixture was

made of equal volumes of solutions (1) and (2). It was prepared immediately before use.

The tyrosine-containing peptide was identified by the staining method described by Easley (1965). A fresh chromatogram without previous staining was dipped through buffered ninhydrin solution (1 ml pyridine and 1 ml glacial acetic acid was added to 98 ml 0.3% ninhydrin in acetone). The dried paper was warmed to 70° or 80°. Ninhydrin spots were circled with a pencil. The chromatogram was dipped through solution A (0.1% α-nitroso-β-naphthol in acetone) and dried, and then through solution B (10 ml conc. HNO_3 plus 90 ml acetone, freshly prepared). The paper was dried for 5 to 10 minutes and then warmed carefully over a hot plate. As the fumes started to rise from the paper, the background changed from dark yellow to light yellow and at the same time, the tyrosine spot turned a rose color. Using the developed strip as marker, the areas of the other chromatograms containing the tyrosine-peptides were cut out, eluted with distilled water for 24 hr, and evaporated to dryness on a rotary evaporator.

E. Amino Acid Analysis

1. Qualitative analysis. The iodotyrosyl-containing peptide isolated from 1.5 mg of thyroglobulin was hydrolyzed in 6 N HCl in a sealed tube for 24 hr at 105° to 110°. After hydrolysis the tube was opened and the HCl

removed in vacuo with a rotary evaporator. The hydrolyzed peptide was dissolved in aliquots of water and applied one inch from the lower corner of a sheet of Whatman No. 1 filter paper. Amino acids were identified by ascending chromatography in the first dimension with n-butanol-pyridine-water (30:30:30 v/v) system and with phenol-ammonium hydroxide (0.88 M) (100:0.5 v/v) in the second dimension. Reference amino acids were run simultaneously on separate chromatograms. The chromatograms were washed with peroxide-free ether and sprayed with 0.5% ninhydrin solution in n-butanol.

2. Quantitative analysis. The amino acids were analyzed by the colorimetric method described by Spies (1957).

a. Reagents.

(1) Ninhydrin solution. Stannous chloride, dihydrate (0.80 g) was dissolved in 500 ml of the 0.2 M citrate buffer. This solution was added to 20 gram of ninhydrin dissolved in 500 ml of cellosolve. The solution was flushed with pure N_2 for about 10 min.

(2) Dilute solvent. Equal volumes of water and reagent grade n-propanol were mixed.

b. Procedure. The ninhydrin spots of each amino acid were cut from the chromatogram and placed in test tubes. One milliliter of ninhydrin was added. The tubes were covered with an aluminum cap and heated for

20 min in a boiling water bath. Nine milliliters of diluent solvent was then added to each tube, and the contents mixed. Readings were taken on a spectrophotometer (Spectronic 20, Bausch & Lomb) at 570 mμ starting 15 min after the tubes had been removed from the water bath. Determinations were also made on a blank piece of paper cut from suitable parts of the chromatogram sheet. A leucine standard curve was prepared over a concentration range from 0.2 to 2.0 mM.

F. Amino Acid Sequence Analysis

1. Determination of the amino-terminal amino acid using the Edman Degradation. The sequence of amino acid residues in the peptide was determined primarily by means of the Edman phenylthiohydantoin (PTH) procedure. The following modification (Hill, et. al., 1969) of the method was routinely used. Dried iodotyrosyl-containing peptide isolated from 5 mg of thyroglobulin in a stoppered small tube was dissolved in 0.1 ml of distilled water and mixed with an equal volume of 1% phenylisothiocyanate (PITC) in pyridine and 0.025 ml of 25% triethylamine. The tube was flushed with pure N_2 for about ten seconds. The mixture was maintained at 40° for 2 hr. Water (0.3 ml) was added, and the sample was extracted with 0.5 ml of water-saturated cyclohexane five times and with 0.5 ml of thiophene-free, water-saturated benzene three times.

The extracts were discarded and the aqueous phase evaporated to dryness in vacuo with a rotary evaporator. For cyclization, 0.2 ml of glacial acetic acid-conc. HCl (5:1 v/v) was added and the solution was incubated at 40° for 1 hour under N_2. The acid was removed under reduced pressure with a rotary evaporator. One-half milliliter of 0.01 N HCl was added and the sample was extracted three times with an equal volume of peroxide-free, water-saturated ether. The ether phase was evaporated in a test tube by placing the tube in a beaker of warm water and directing a stream of pure N_2 over the surface. The PTH amino acid was dissolved in 0.1 ml of acetone. Reference and the unknown PTH amino acids were spotted on Whatman No. 1 filter paper (10 x 10 inch) along a line one inch from the edge of the paper. Ascending chromatography in a solvent system of n-heptane-pyridine (6:4 v/v) (Edman, 1950) was carried out for 2 to 3 hr. The chromatograms were dried in a current of air and steamed with a vigorous jet until all of the smell of pyridine had gone. The PTH amino acids were identified directly either by short wave ultra-violet light or by spraying with iodine-azide-starch reagent (Sjoquist, 1953) prepared as follows: (1) Iodine, 2.54 gram and 8 gram of KI, in water made up to 100 ml; (2) 0.5 gram of soluble starch mixed into a smooth paste with 3 to 4 ml of cold water and then poured into 90 ml boiling water. The solution was allowed to cool , 1.5 gram

of sodium azide was added, and then made up to 100 ml. One volume of solution (1) and two volumes of solution (2) were mixed as required.

2. Determination of the amino-terminal amino acid using dinitrophenyl (DNP) method. Using the dinitrophenyl (DNP) method to determine the N-terminal residues, the following procedure (Clark, 1963) was used. Dried iodotyrosyl-containing peptide isolated from 1.5 mg of thyroglobulin was dissolved in 1 ml of 2% sodium bicarbonate solution and mixed with 2 ml of ethanol containing 0.05 ml of 1-fluoro-2,4-dinitrobenzene (FDNB). This mixture was shaken, in the dark, at room temperature for 5 hours. At the end of this time the solution was diluted with 5 ml of distilled water and extracted three times with equal volumes of peroxide-free ether. After acidification of the aqueous phase with two drops of 6 N HCl, the DNP-peptide was extracted with two 2 ml portions of ether. The combined ether extracts were washed with 0.01 N HCl and then evaporated to dryness in a test tube by placing the tube in a beaker of warm water and directing a stream of air over the surface. The dried DNP-peptide was dissolved in 6 N HCl and transferred to a small vial. The vial was sealed and placed in an oven at 105° for 24 hr. After hydrolysis, 2 ml of water was added and the mixture was extracted with 2 ml of peroxide-free ether three times. The combined ether extracts were concentrated as

before. The DNP amino acid was dissolved in 0.1 ml of acetone, and spotted on Whatman No. 1 filter paper (10 x 10 inch). Ascending chromatography was carried out in n-butanol saturated with ammonia (0.1%) for 8 hr. Standard DNP amino acids were prepared in the same way and developed simultaneously with the unknown DNP amino acid. The DNP amino acids can be located by their ultra-violet absorption.

3. Determination of the carboxyl-terminal amino acid using carboxypeptidase A. The C-terminal residues can be determined by hydrolysis with carboxypeptidase A. The following method by Jackson and Hirs (1970) was used, except that the enzyme reaction was carried out at 38°C rather than 25°C. Dried iodotyrosyl-containing peptide isolated from 1.5 mg of thyroglobulin was dissolved in 0.10 ml of 1 M NaCl and the solution was diluted with 0.20 ml of 0.1 M Tris-HCl buffer, pH 8.0. A 0.4% solution of carboxypeptidase A in the same buffer was prepared and the digestion was started by addition of 0.01 ml of this enzyme solution to the peptide solution at 38°. Aliquots of 50λ were removed at 0, 0.5, 1, 2, 4, and 6 hours. The reaction was stopped by the addition of 0.025 ml of glacial acetic acid. The solution was evaporated to dryness in a rotary evaporator and the residue was analyzed by ascending chromatography in n-butanol-acetic acid-water (40:6:15 v/v) and stained with 0.5% ninhydrin in n-butanol.

IV. RESULTS

A. Isolation and Identification of Iodotyrosyl-Containing Peptide

An iodopeptide produced by tryptic digestion of thyroglobulin was separated by high voltage paper electrophoresis in pyridine-acetate buffer (pH 6.8), as indicated in Fig. 1. This tryptic fraction which contained the iodopeptide migrated toward the cathode. Since this fraction containing the iodopeptide appeared as a yellow color on the chromatogram under ultra-violet light, it was not necessary to develop a ninhydrin stained marker. Although a single spot migrated to the cathode, it contained a number of other tryptic fragments. In order to isolate the iodopeptide, the tryptic fractions were further purified by paper chromatography with the solvent system of n-butanol-acetic acid-water (4:1:1 v/v). The separated peptide map is shown in Fig. 2. The iodopeptide remained at the origin. Other tryptic fragments moved to higher positions.

The iodopeptide eluates were chromatographed in a solvent system of ethanol-ammonium hydroxide (0.88 M)-water (90:5:5 v/v). The dried chromatograms were treated with ceric-arsenite reagent and stained with ninhydrin-α-nitroso-β-naphthol solution separately as described under "Experimental". The results are shown in Fig. 3. There are two

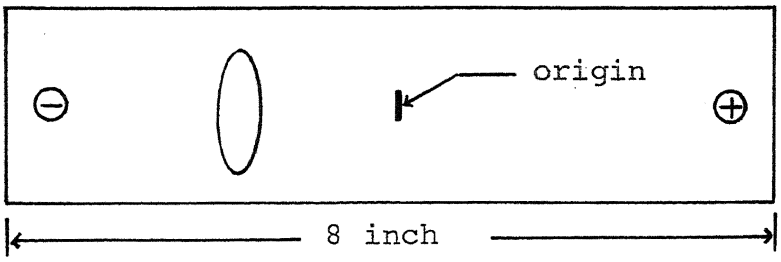


Figure 1. Paper Electrophoresis of Thyroglobulin Digested with Trypsin.
(Electrophoresis was carried out in pyridine-acetate buffer, pH 6.8, at 10 to 15 V/cm for 4 hr. Spot was observed under ultra-violet light.)

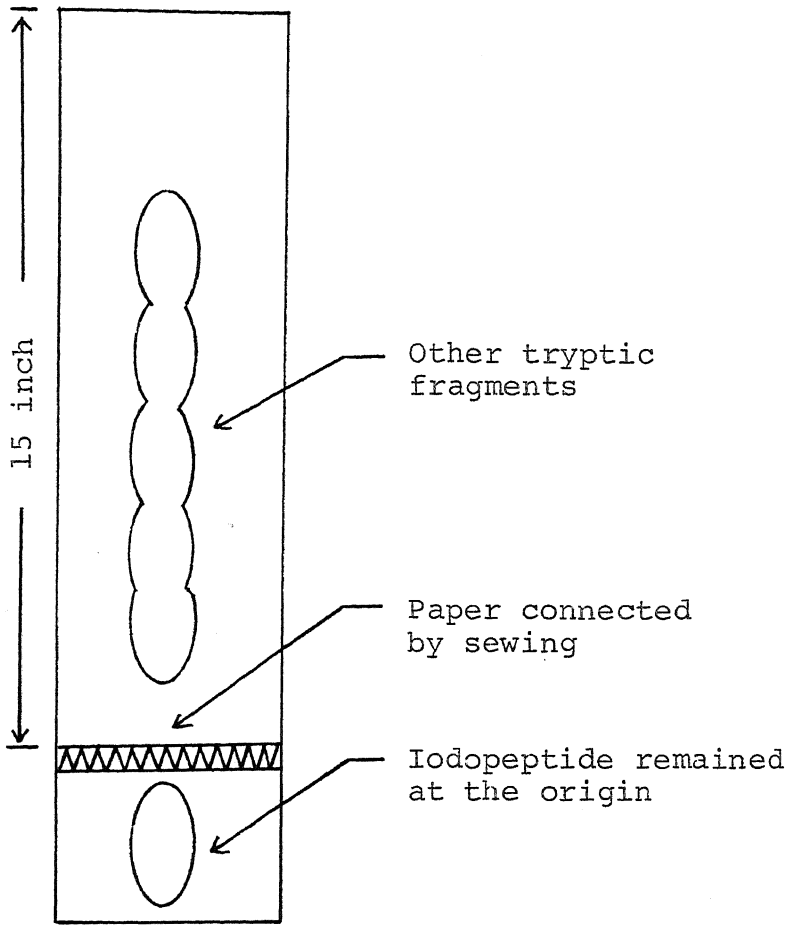


Figure 2. Separation of Iodopeptide by Paper Chromatography. (The chromatograms were hung in the chamber and developed by ascending chromatography in a solvent system of n-butanol-acetic acid-water (4:1:1 v/v) for 18 hr.)

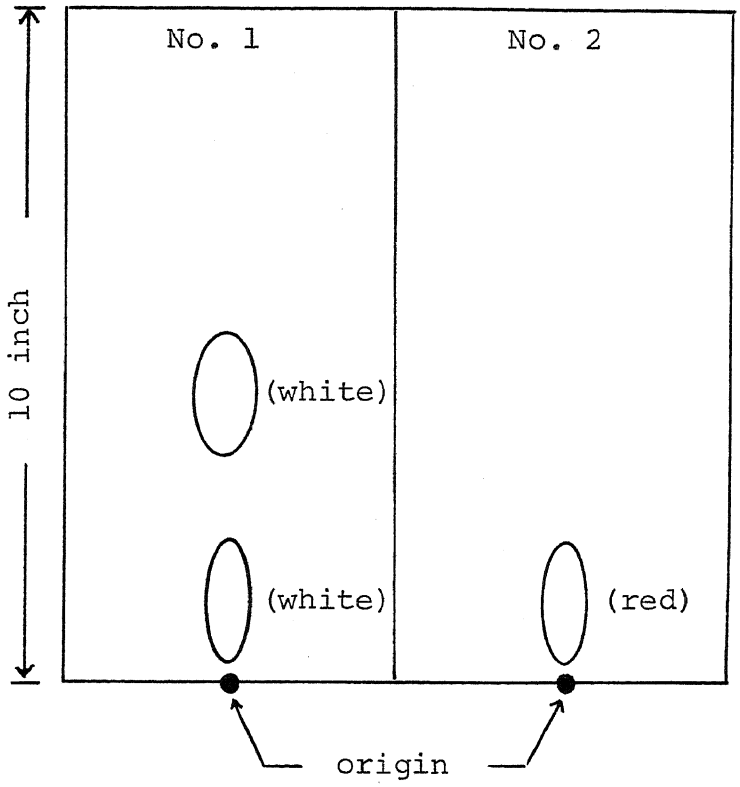


Figure 3. Identification of Iodotyrosyl-Containing Peptide.

(Ascending chromatography in a solvent system of ethanol-ammonium hydroxide (0.88 M)-water (90:5:5 v/v) for 6 hr. No. 1 chromatogram was treated with ceric-arsenite reagent; No. 2 chromatogram was stained with ninhydrin-α-nitroso-β-naphthol solution.)

white spots which appear on the ceric-arsenite treated chromatogram, while only the lower spot exhibits a positive reaction to the ninhydrin-α-nitroso-β-naphthol reagent. This indicates that the lower spot is the iodotyrosyl-containing peptide.

B. Amino Acid Composition

The amino acid composition of the iodotyrosyl-containing peptide is given in Table III. The amino acids were identified by paper chromatography and the concentration of each was estimated by the colorimetric method. Paper chromatography of the acid hydrolysate is shown in Fig. 4. The solvent pair was satisfactory for the separation of these amino acids, except for spots No. 7 and No. 8. It is difficult to distinguish whether No. 7 is methionine or leucine (isoleucine). For this purpose, hydrogen peroxide oxidation was used. One-tenth ml of cold 30% H_2O_2 solution was added to the acid hydrolysates and standard methionine. After standing 1 to 2 minutes, oxidation of sulphur compounds was complete and the solution was then chromatographed. The results indicated that standard methionine disappeared while the No. 7 spot was still present. Therefore, the No. 7 spot is confirmed as isoleucine (or leucine). The No. 8 spot due to its more bluish ninhydrin color, is confirmed as phenylalanine. Since leucine and isoleucine have similar Rf values, no attempt was

TABLE III

AMINO ACID COMPOSITION OF IODOTYROSYL-CONTAINING PEPTIDE[a]

Amino Acid	Molar Ratio[b]
Aspartic acid	1.17 (1)
Glutamic acid	1.05 (1)
Serine	1.06 (1)
Glycine	1.16 (1)
Alanine	1.55 (2)
Tyrosine	1.00 (1)
Isoleucine (Leucine)	1.07 (1)
Phenylalanine	1.31 (2)[c]

a. The composition of the peptide is given as the molar ratios of the amino acids, calculated without corrections for destruction during acid hydrolysis and for color yield.

b. Molar ratio was calculated on the assumption that the peptide contained one tyrosyl residue.

c. Assumed value.

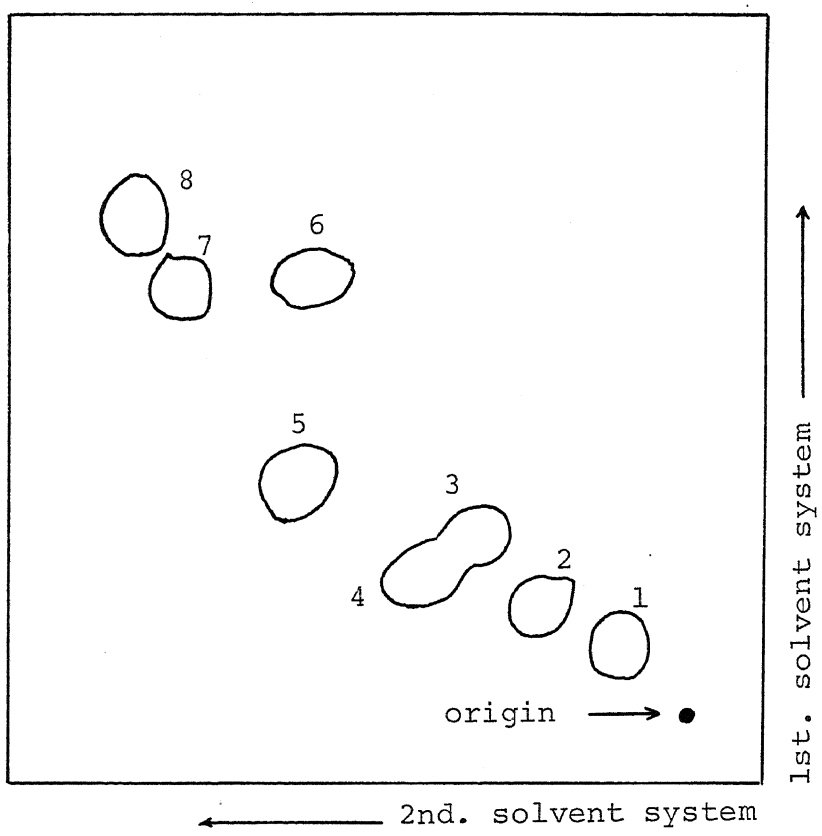


Figure 4. Paper Chromatography of the Acid Hydrolysate.

(Iodotyrosyl-containing peptide which was hydrolyzed with 6 N HCl at 105 to 110° for 24 hr. 1st solvent system: n-butanol-pyridine-water (30:30:30 v/v), 2nd solvent system: phenol-ammonium hydroxide (0.88 M) (100:0.5 v/v). Development time: 10 hrs. Spots were stained with 0.5% ninhydrin in n-butanol.)

made to distinguish between these two. The Rf values of each amino acid is given in Table IV.

The amino acid concentrations were obtained from the standard leucine curve shown in Fig. 5. This curve followed Beer's law through an optical density of 0.5. This peptide contained 1.31 moles of phenylalanine, which was assumed to represent 2 residues. This was probably due to the low color yield of phenylalanine.

C. Sequence Analysis

Application of the dinitrophenylation procedure to the iodotyrosyl-containing peptide revealed the presence of a single NH_2-terminal residue, alanine (Fig. 6). Although two spots appeared on the chromatogram, the slower moving spot was dinitrophenol and the faster one was the true DNP amino acid.

The sequence of amino acids 1 through 6 in the peptide was determined by the Edman procedure as indicated in Fig. 7. Although an attempt was made to extend the sequence, no spot appeared on the chromatograms of the seventh or ensuing degradations. In the fourth degradation step, two spots appeared. The lower spot is PTH-serine, however, the higher one is probably PTH-Δ-serine (Yang, et. al., 1969). Although the PTH-serine and PTH-tyrosine have similar Rf values, a tyrosyl residue was released by carboxypeptidase A in the carboxyl-terminal residue determination. Since

TABLE IV

THE R_f VALUES OF AMINO ACIDS PRESENT IN THE IODOTYROSYL-CONTAINING PEPTIDE

Spot No.	Amino Acid	1st. solvent	2nd. solvent
1	Aspartic acid	0.135	0.124
2	Glutamic acid	0.171	0.233
3	Serine	0.279	0.321
4	Glycine	0.243	0.400
5	Alanine	0.360	0.570
6	Tyrosine	0.640	0.549
7	Isoleucine (Leucine)	0.587	0.720
8	Phenylalanine	0.666	0.772

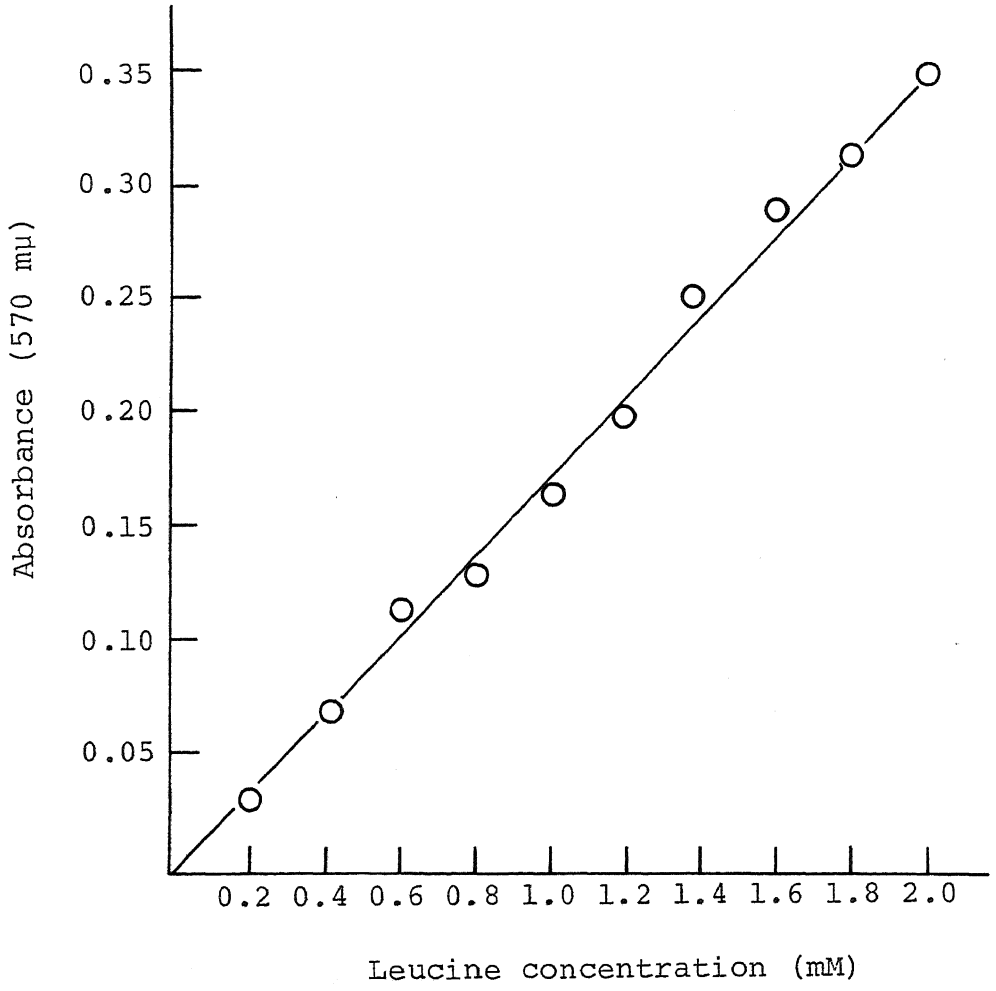


Figure 5. Quantitative Ninhydrin Standard Curve.

(Conditions as described on page 20).

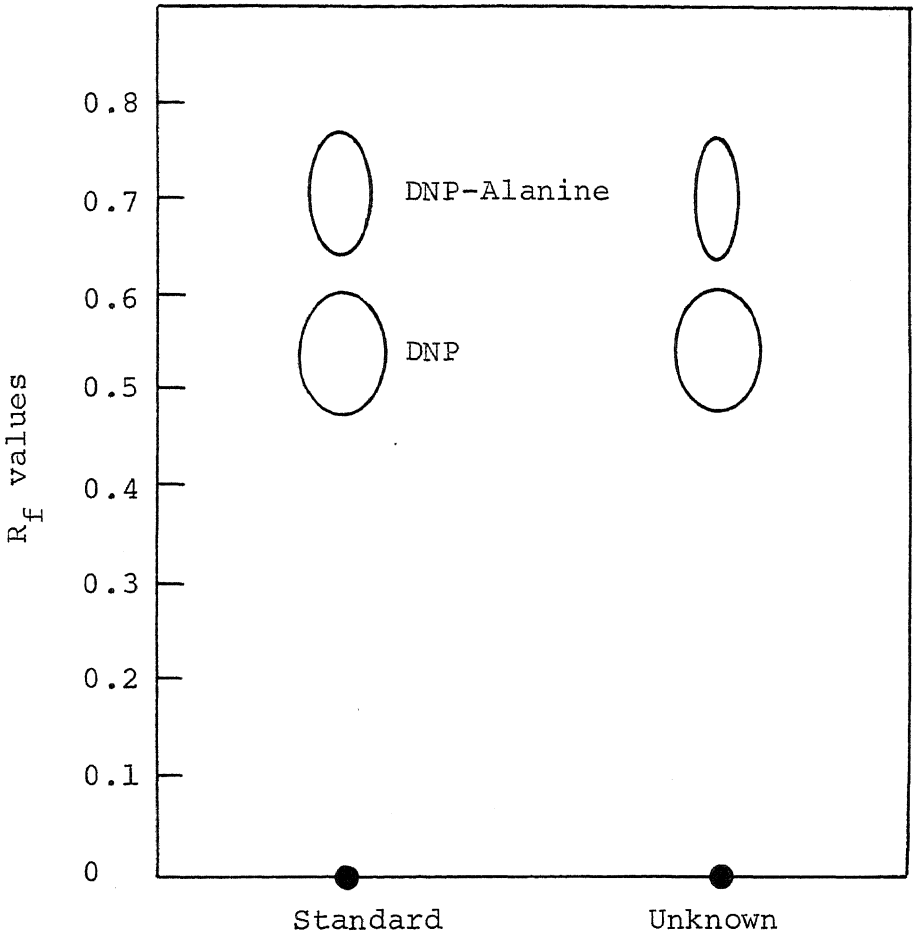


Figure 6. Paper Chromatography of Amino-Terminal DNP-Amino Acid.

(Solvent system: n-butanol saturated with ammonia (0.1% w/v). Development time: 8 hr. Spots were examined under ultra-violet light.)

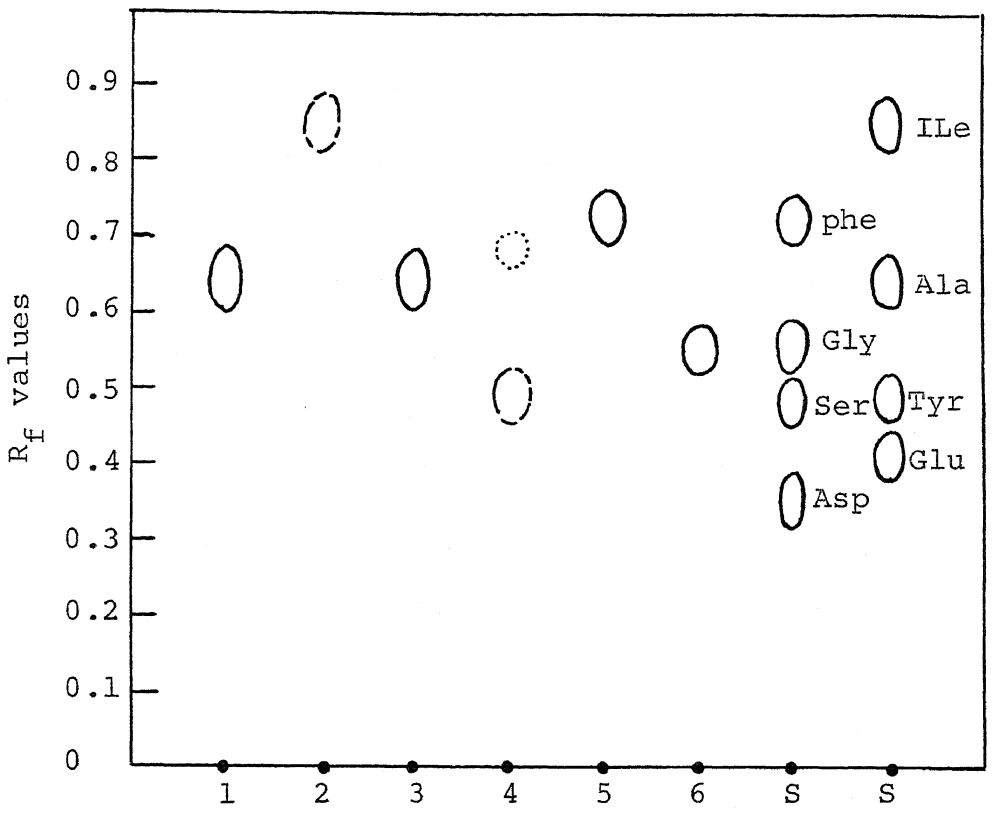


Figure 7. Composite Drawing of Chromatogram for Products from Six Degradations of Iodotyrosyl-Containing Peptide.

(Ascending chromatography in a solvent system of n-heptane-pyridine (6:4 v/v) for 2.5 hr. Spots were identified either by ultra-violet light or by spraying with iodine-azide-starch reagent. Quantity: _____, Spot prominent;-----, Spot Apparent;....., Spot only faintly visible. S: the mixture of standard PTH amino acids; the number refers to the order of degradation.)

only one tyrosyl residue is present in the peptide, the fourth residue is confirmed as serine.

A carboxyl-terminus determination using carboxypeptidase A is shown in Fig. 8. The results were less satisfactory than the Edman procedure. This enzyme released phenylalanine first and trace quantities of glutamic acid and tyrosine. The intensity of the color with ninhydrin was so faint that the sequence was difficult to determine. Since it is known that carboxypeptidase A releases tyrosyl residues more readily than it does glutamyl residues, it would appear that the C-terminal sequence is as shown in Fig. 9. If a tyrosyl residue is adjacent to the carboxyl-terminal residue, phenylalanine, it should have a higher intensity of ninhydrin color. Furthermore, the two acidic residues (aspartic acid and glutamic acid) clustered together would have prohibited the liberation of glutamyl residue by carboxypeptidase A. Although the glutamyl residue is resistant to carboxypeptidase A, it still can be released slowly. Consequently, the results suggest that the glutamyl residue is adjacent to the carboxyl-terminus phenylalanine. Prolonged hydrolysis increases the color intensity of the phenylalanyl residue but gave no further residues. It is implied that the next residue adjacent to the tyrosyl residue is aspartic acid which is strongly resistant to carboxypeptidase A. The complete sequence of this iodotyrosyl-containing peptide is given in Fig. 9.

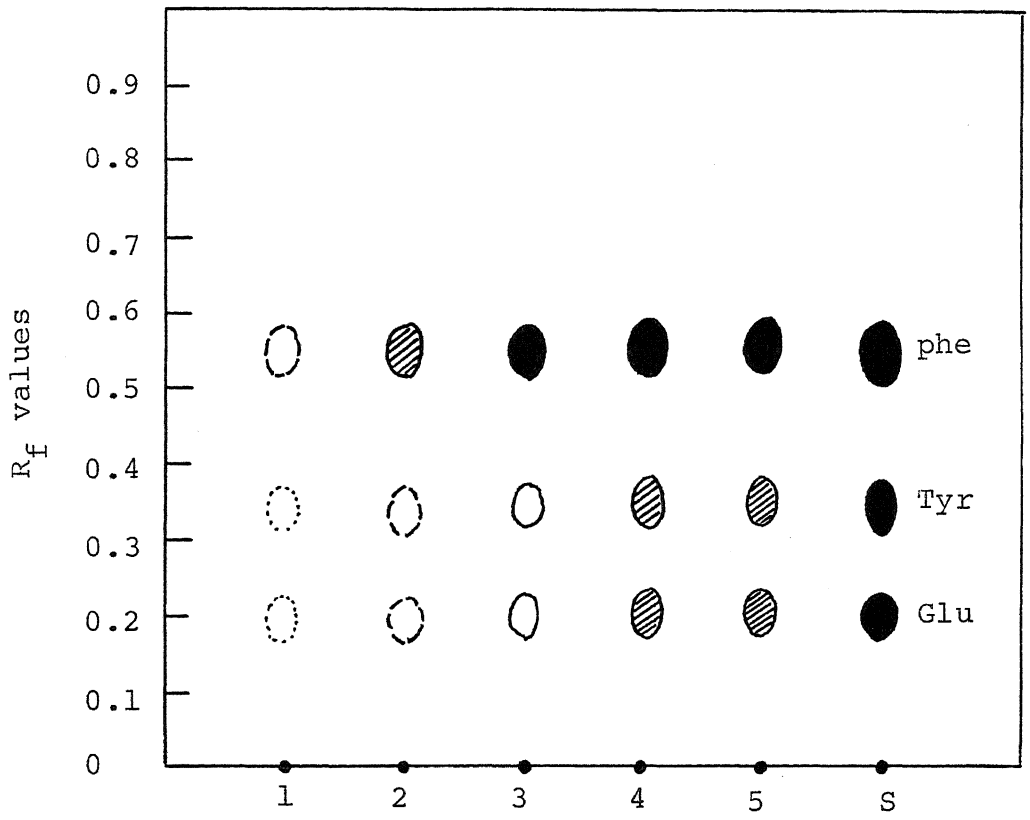


Figure 8. Paper Chromatography of Carboxypeptidase A Digests.

(The enzyme reaction was carried out in 0.1 M Tris-HCl buffer (pH 8.0) at 38°. Aliquots were removed and acidified at 0.5 hr (1), 1 hr (2), 2 hr (3), 4 hr (4), and 6 hr (5). S: mixture of standard amino acid. Chromatograms were developed in n-butanol-acetic acid-water (40:6:15 v/v) for 10 hr and stained with 0.5% ninhydrin in n-butanol.)

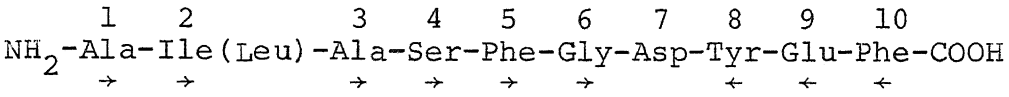


Figure 9. The Complete Amino Acid Sequence of the Iodotyrosyl-Containing Peptide.

(Sequence determined by the Edman technique, is shown by → , from the N-terminal end. Sequence determined hydrolysis with carboxypeptidase A, shown by ← .)

V. DISCUSSION

There are several interesting features in the amino acid sequence of this iodotyrosyl-containing peptide. It contains two acidic residues (aspartic acid and glutamic acid) and lacks any basic amino acid residues. Because of its high content of acidic residues and the absence of basic amino acids such as lysine and arginine, its isoelectric point (pH_i) will be in the acidic region. The isoelectric point of thyroglobulin has been determined by Heidelberger and Pedersen (1935) as approximately 4.6. From the amino acid composition of thyroglobulin (Peiz and Morris, 1960), it may be noted that acidic residues (aspartic acid and glutamic acid) exceed the content of the basic residues (lysine and arginine). This accounts for the acid isoelectric point of thyroglobulin.

Trypsin was chosen for the hydrolysis of thyroglobulin because of its specificity. However, the release of this iodotyrosyl-containing peptide by trypsin did not produce the expected cleavage at the peptide bonds adjacent to the carboxyl group of the lysyl and arginyl residues. This peptide having a carboxyl-terminal phenylalanine residue may represent a C-terminal peptide of thyroglobulin or a lack of specificity of the trypsin. This abnormal behavior of trypsin may be explained either by the possibility that crystalline trypsin used in this study was a

commercial preparation containing some chymotryptic activity or by an inherent chymotryptic action of trypsin (Inagami and Sturtevant, 1960). In either case, this action renders it difficult to conlude that any of the isolated peptides represent authentic tryptic fragments even when lysine or arginine is in the COOH-terminal position.

Another interesting feature of this amino acid sequence is the two acidic residues are concentrated on the side toward the carboxyl-terminal end of the chain and the hydrophobic residues are clustered mainly near the NH_2-terminal region of the peptide chain. The hydrophobic residues, amino acids having long chain aliphatic or aromatic side chains appear to be in the region represented by Residues 1 to 5. The polar residues are located in the region of Residues 7 to 9. The tyrosyl residue is in the less hydrophobic or more aqueous environment. Although no evidence from x-ray studies is available, this tyrosyl residue is probably on the surface of the thyroglobulin molecule and, hence, exposed to solvent.

Probably the most interesting facet of the peptide structure is the possibility that either one or both of the acidic amino acids could serve as a recognition site for an iodinase enzyme. In this manner, it might then be possible to explain why only a very few of the tyrosyl residues in thyroglobulin are iodinated. Edelhoch and

Perlman (1968) used N-acetyl imidazole to assess the reactivity of tyrosyl residues present in native thyroglobulin and in human serum albumin. They found that the reactivity of the tyrosyl residues in the two proteins was different. In human serum albumin the residues that react with N-acetyl imidazole are largely unreactive with iodine. In thyroglobulin the residues that are acetylated are the first to be iodinated. This difference in tyrosyl residue behavior may be related to the environment of the residues in the two proteins. The first tyrosyl residues that are iodinated in human serum albumin (Perlman and Edelhoch, 1967) show important change in their ultraviolet difference absorption spectrum in 8 M urea as compared to that in H_2O at pH 3.9 which are indicative of their being in a hydrophobic environment. However, only relatively small difference absorption spectrum in 7.8 M urea as compared to 2 M urea at pH 3.5 occur when thyroglobulin is iodinated. Consequently, it appears that the reactive tyrosyl groups in thyroglobulin are in a hydrophylic or more polar environment than those in human serum albumin. In the present study, we have found one of the tyrosyl residues of thyroglobulin to be in a hydrophylic environment.

It is known that the iodination of tyrosyl groups of thyroglobulin is regulated by the secondary and tertiary structures. Metzger, et. al., (1962) found that fragments

from the tryptic digestion showed no secondary or tertiary structure when tested by the standard procedures. If the tyrosyl residue studied in this work is assumed to be on the surface of the thyroglobulin molecule and exposed to the solvent, and since it has been shown that the hydroxyl group is in a hydrophilic environment, it should be ionized normally, and, hence, be iodinated easily. If the opposite assumption is made, i.e., the tyrosyl residue is embedded in the peptide chain, an intramolecular non-covalent bond, tyrosyl-carboxyl bond (i.e., side chain hydrogen bonding OH^-.....COOH) probably occurs, and the hydroxyl group of the tyrosyl residue will be ionized abnormally. The abnormally ionized tyrosyl residue cannot be iodinated. These assumptions are supported by the studies on the iodination of ribonuclease A by Cha and Scheraga (1963). They found that four of the six tyrosyl residues were converted to a mixture of monoiodotyrosyl and diiodotyrosyl residues and the two abnormally ionized tyrosyl residues were uniodinated.

Finally, although knowledge of the amino acid sequence of iodotyrosyl-containing peptide produced by tryptic digestion of thyroglobulin does not indicate the degree of iodination of the tyrosyl residue, it does provide some clues as to possible configuration and a basis upon which further studies may yield an understanding of the interrelationship of primary structure and function of thyroglobulin.

VI. CONCLUSIONS

A single iodotyrosyl-containing peptide was isolated from tryptic digestion of thyroglobulin. The amino acid composition of this peptide was identified as aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine, isoleucine (leucine), and phenylalanine. The amino acid sequence of this peptide is:

NH_2-Ala-Ile(Leu)-Ala-Ser-Phe-Gly-Asp-Tyr-Glu-Phe-COOH

The position of tyrosyl residue is in a less hydrophobic environment, suggesting that this tyrosine is normally exposed to the aqueous environment. Under such conditions the phenolic group would be ionized and iodination enhanced. The amino acid sequence of this iodotyrosyl-containing peptide would appear to play an important role in the iodination process.

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VIII. VITA

Pai-Chun Chiang was born on January 18, 1939 in Taiwan. He attended high school in Tainan, Taiwan, and graduated in June of 1957. He entered the National Taiwan University, Taipei, Taiwan in the fall of 1957 and received the degree of B.S. in Agricultural Chemistry in June 1961. He then served in the Republic of China Army for one year, and worked at Wei-Shin Foods Corporation, and the United States Naval Medical Research Unit No. 2 in Taipei.

He came to the United States in September 1967 and enrolled at the University of Missouri-Rolla as a graduate student in Chemistry.